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Host-specific serological response to *Angiostrongylus vasorum* infection in red foxes (*Vulpes vulpes*) and impact of heat treatment on antigen detection in dog sera

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Summary

Angiostrongylus vasorum is a cardiopulmonary nematode increasingly found in dogs and foxes in many European countries. ELISAs for the detection of circulating *A. vasorum* antigen or specific antibodies in dogs were evaluated for foxes. Blood from 75 farmed Danish foxes experimentally infected with *A. vasorum* and from 215 dissected wild Swiss foxes were evaluated for detection of circulating *A. vasorum* antigen and specific antibodies. Antigen detection had 91.2% sensitivity and 89.4% specificity, and antibody detection 42.2% and 92.0%, respectively. The experimentally infected foxes became antigen positive 5 to 10 weeks post inoculation (wpi) and remained positive up to 22 wpi. The antibody responses in the same foxes were highly variable, irrespectively of further challenge inoculations, reaching seropositivity 5 to 7 wpi and followed by a decrease in over half of the animals despite persistent infections. Infected foxes may develop a variable and non-protective immunity which contributes to long term survival of *A. vasorum* that could explain its efficient spread within the fox population. Furthermore, the effect of EDTA/heat treatment on *A. vasorum* antigen detection in dog sera was assessed with 205 sera before and after treatment. An improvement of 34.6% was observed in treated samples between 7 and 10 wpi, while detection was impaired between 3 and 5 wpi. The same procedures applied to 74 samples tested with a rapid assay did not substantially improve antigen detection.

Zusammenfassung

Angiostrongylus vasorum ist ein kardiopulmonaler Nematode, welcher vermehrt in Hunden und Füchsen in Europa diagnostiziert wird. ELISAs für den Nachweis von zirkulierenden *A. vasorum* Antigenen oder spezifischen Antikörpern in Hunden wurden für Füchse evaluiert. Blut von 75 experimentell mit *A. vasorum* infizierten dänischen Zuchtfüchsen und von 215 sezierten Schweizer Füchsen wurde ausgewertet. Der Antigennachweis hatte 91.2% Sensitivität und 89.4% Spezifität, der Antikörpernachweis jeweils 42.2% und 92.0%. Experimentell infizierte Füchse wurden 5 bis 10 Wochen post Inokulation (wpi) Antigen-positiv und blieben bis 22 wpi positiv. Die Antikörperantwort in den gleichen Füchsen war stark variabel, unabhängig von weiteren Inokulationen. Seropositivität trat nach 5 bis 7 wpi auf, gefolgt von einer Abnahme in über der Hälfte der Tiere trotz persistierender Infektionen. Infizierte Füchse entwickeln eine variable, nicht-protektive Immunität, welche zum Langzeitüberleben von *A. vasorum* und somit zur Erklärung für dessen effiziente Verbreitung beiträgt. Ferner wurde der Effekt von EDTA/Hitzebehandlung auf den *A. vasorum* Antigennachweis in 205 Hundeseren vor und nach Behandlung evaluiert. Eine Antigennachweissteigerung in behandelten Seren um 34.6% zwischen 7 und 10 wpi wurde beobachtet, während zwischen 3 und 5 wpi eine Abnahme auftrat. Dieselbe Vorgehensweise führte bei 74 mit einem Schnelltest getesteten Seren zu keiner wesentlichen Verbesserung des Antigennachweises.

Host-specific serological response to *Angiostrongylus vasorum* infection in red foxes (*Vulpes vulpes*): implications for parasite epidemiology

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SUMMARY

Angiostrongylus vasorum is a cardiovascular nematode increasingly found in dogs and foxes in endemic foci throughout Europe. The present study evaluates ELISAs for detection of circulating antigens and specific antibodies against *A. vasorum* in foxes. Blood and worm burdens (WBs) from carcasses of 215 Swiss wild red foxes (*Vulpes vulpes*) and from 75 farmed foxes of different age groups experimentally inoculated once or repeatedly with infective doses of 50, 100 or 200 third-stage larvae were obtained. Antigen detection in the naturally infected Swiss foxes had 91·2% sensitivity and 89·4% specificity, whereas the corresponding figures for antibody detection were 42·2 and 92·0%. The experimentally infected foxes became positive for circulating antigens 5–10 weeks post-inoculation (wpi) and remained highly positive up to 22 wpi, irrespectively of further challenge inoculation. The antibody responses in the same foxes were highly variable: high optical density (OD) values were reached 5–7 wpi in all animals, followed by a decrease in over half of the animals despite accumulating and consequently high WBs resulting in persistent infections. After each challenge, a slight increase of OD values was observed 7 weeks later. We hypothesize that infected foxes develop a variable and non-protective immunity. Such parasite tolerance allows long-term survival of *A. vasorum* in the animals, and may explain why the parasite appears to spread rapidly within a fox population, an epidemiological dynamic that is evident in many parts of Europe where *A. vasorum* has been found over the last decades.

Key words: *Angiostrongylus vasorum*, foxes, ELISA, antigen, antibody, sensitivity, specificity, immune response, serology, lungworm.

INTRODUCTION

Angiostrongylus vasorum (Baillet, 1866) is a nematode that lives in the pulmonary arteries and the right heart of foxes, dogs and other canids. Slugs and snails are the relevant intermediate hosts (Guilhon and Cens, 1973) harbouring the infective third-stage larvae (L3) that are orally ingested by the definitive host.

Reports from several European countries show that *A. vasorum* has established and is wide-spread in the fox population. Over the last decades, increasing local and regional prevalence (Saeed *et al*. 2006; Morgan *et al*. 2008; Al-Sabi *et al*. 2013; Eleni *et al*. 2014; McCarthy *et al*. 2016) and accumulating reports of new endemic areas indicate an increased transmission and apparent geographical spread (Sréter *et al*. 2003; Taylor *et al*. 2015). Respiratory signs, coagulation disorders and occasionally neurological symptoms are typically occurring in dogs with angiostrongylosis (Chapman *et al*. 2004; Schnyder *et al*. 2010), and if left untreated, the disease may be fatal. On the contrary, descriptions of clinical signs in foxes are rare. Simpson (1996) described clinical illness in two shot foxes, diagnosed with severe *A. vasorum* infection at necropsy, including aimless wandering and emaciation. In naturally infected foxes, description of right ventricular hypertrophy, granulomatous pneumonia and arterial media hypertrophy may further suggest that pathological changes can be severe and eventually lead to respiratory failure and death (Poli *et al*. 1991; Eleni *et al*. 2014). In opposition to that, studies from UK and Newfoundland (Canada) showed that body condition was not different in naturally infected and uninfected foxes (Jeffery *et al*. 2004; Morgan *et al*. 2008). Also in experimentally infected foxes clinical signs were absent within the first 10–20 weeks of infection (Webster *et al*. 2017; Woolsey *et al*. 2017). Based on their widespread occurrence and the high prevalence of *A. vasorum* in foxes, the observed limited impact of *A. vasorum* infection on fox health and on the fact that they are not, in

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general, subject to anthelmintic treatment, it is assumed that foxes represent the most important definitive host responsible for parasite transmission.

In dogs, the most common diagnostic method is the isolation of *A. vasorum* first-stage larvae (L1) from fecal samples (Deplazes *et al*. 2016), or, increasingly, by serological tests (Schnyder *et al*. 2015). Also, a bedside clinical assay for rapid diagnosis detecting antigen is available (Schnyder *et al*. 2014). In foxes, the detection of *A. vasorum* is most often accomplished by recovery of adult specimens from lungs during necropsy (Saeed *et al*. 2006; Magi *et al*. 2009; Taylor *et al*. 2015), or by isolation and PCR typing of fecal larvae (Al-Sabi *et al*. 2010). For epidemiological investigations, a serological test applicable on blood and/or tissue fluids would allow for a faster and less labour intensive method than dissection and therefore would be more appropriate for mass-screening of fox samples. Our aim was to evaluate enzme-linked immunosorbant assays (ELISAs) for detection of circulating *A. vasorum* antigen (Ag-ELISA) and specific antibodies (Ab-ELISA) in sera/blood from naturally and experimentally infected foxes.

MATERIALS AND METHODS

Necropsy and sample collection of wild foxes

Over the years 2013–2015, 215 Swiss red foxes (*Vulpes vulpes*) from the northeastern and eastern part of Switzerland were shot by hunters during the hunting season and provided for endoparasite examination to the Institute of Parasitology, University of Zurich. The content of the intestines was examined macroscopically and by sedimentation to identify intestinal parasites, as previously described (Hofer *et al*. 2000). Lungs and hearts were finely dissected along the blood vessels and airways, and repeatedly washed in a water filled funnel glass. The sediment was then examined for lungworms, including *A. vasorum*, and the worm burden (WB) was determined. Sex and approximate age of foxes (younger or older than 1-year old) based on tooth wear and morphology were determined and blood was collected either from the heart or from the bag in which the heart and lung tissues were stored.

Serum samples from experimentally inoculated silver foxes (V. vulpes)

The serum samples originated from two previously performed experiments (Webster *et al*. 2017; Woolsey *et al*. 2017; for the experimental design: see Supplementary Figs. S1 and S2). Briefly, in the first experiment (trial 1) 28 female foxes, comprising 14 adults (>1·5 years old) and 14 juvenile foxes (5 months old), were inoculated with *A. vasorum* L3 originating from experimentally infected *Biomphalaria glabrata* snails. The L1 used to infect *B. glabrata* were isolated from feces of a naturally infected Danish dog and passaged once in farmed foxes. Half of each age group was infected with 50 L3, the other half with 200 L3. In the second experiment (trial 2), 48 foxes assigned to five different groups were inoculated with 100 *A. vasorum* L3 once, twice or three times. Fecal samples were collected for determination of larvae per gram feces (LPG). Serum samples were regularly obtained from the jugular vein and WB was determined at necropsy after 9 weeks (trial 1) and 22 weeks (trial 2). These studies were conducted under the Danish experimental animal licence no. 2005/561–1060.

Serological tests

In order to evaluate the quality of the whole blood samples or bloody fluids of hunted foxes and their adequacy for tests originally developed for serum samples, we performed an ELISA for detection of antibodies against *Toxocara canis*, a very common parasite in red foxes. Ten fox whole-blood samples from Ohio (USA) from 1991, 15 fox whole blood samples from Zurich (Switzerland) from 1998, 20 *A. vasorum* positive and 20 negative fox whole-blood samples (as determined by necropsy) from 2013, two samples of dogs experimentally infected with *A. vasorum* as positive controls, as well as 10 sera of specific pathogen-free (SPF) dogs as negative controls were tested. The test was conducted according to the method of Fahrion *et al*. (2008).

The Ag-ELISA was conducted as previously described for dogs (Schnyder *et al*. 2011). For antibody detection, the sandwich-ELISA based on *A. vasorum* adult somatic antigen purified with monoclonal antibodies (mAb 5/5) was used (Schucan *et al*. 2012). Data are presented as individual optical density (OD) values.

Cut-off determination

Cut-off values were determined based on the mean plus three times the standard deviation (S.D.) of the OD values of 45 blood samples (15 randomly selected samples from each of the years 2013, 2014 and 2015) from wild Swiss red foxes negative for *A. vasorum* at necropsy, resulting in a cut-off OD of 0·260 and of 0·144 for the Ag-ELISA and Ab-ELISA, respectively.

Sensitivity and specificity, cross-reactivity

Sensitivity was calculated based on matched necropsy (detection of adult *A. vasorum* specimens or not) and ELISA results of the 215 wild foxes. Additional calculations were performed by combining Ag-ELISA and Ab-ELISA results. Two

different combinations were adopted: In the 'AND' case, only if a sample was positive in both the Ag-ELISA and Ab-ELISA, it was considered positive; if either antigen or antibody results were negative, the sample was considered negative. In the 'OR' case, a positive result in either the Ag-ELISA or Ab-ELISA was considered positive. If both results were negative, the sample was considered negative. Sensitivities and specificities were calculated for both combinations based on the calculated sensitivities and specificities of the Ag-ELISA and Ab-ELISA (Schnyder *et al*. 2013*a*).

Cross-reactivity was evaluated based on ELISA results of 103 *A. vasorum* free wild foxes from the above Swiss survey positive for *Capillaria aerophila* (syn. *Eucoleus aerophilus*) ($n = 88$), *Crenosoma vulpis* ($n = 15$), *T. canis* ($n = 39$), *Echinococcus multilocularis* ($n = 63$), *Taenia* spp. ($n = 27$), *Mesocestoides* spp. ($n = 40$) and/or other nematodes ($n = 11$) upon necropsy.

Statistical analysis

Statistical analysis was performed with Microsoft Windows Excel 2007 and IBM SPSS Statistics 22. Larval output data were log-transformed prior to analysis due to non-normal data distribution. Pearson correlations were calculated for LPG, WB and Ag-ELISA and Ab-ELISA OD values for experimentally infected foxes. In addition, Pearson correlations for WB and antigen and antibody OD values were calculated for the 215 wild Swiss foxes.

RESULTS

Antigen detection in necropsied wild foxes

A total of 102 wild foxes were harbouring adult *A. vasorum* stages detected at necropsy with a WB ranging from 1 to 42 adult specimens (mean = 6·17, median = 3·5, S.D. = 6·93). Of these, 93 foxes (91·2%) were positive for antigen detection. The nine negative foxes in the Ag-ELISA were all harbouring a single adult *A. vasorum* specimen each. Twelve of 113 foxes (10·6%) negative at necropsy were found to be antigen positive. Sensitivity and specificity was 91·2 and 89·4%, respectively, using necropsy data as reference standard. Of 103 foxes harbouring other parasites than *A. vasorum*, ten foxes (9·7%) had antigen values above the cut-off: one infected with *C. aerophila*, two with *Mesocestoides* sp. and seven with mixed endoparasitic infections (Fig. 1).

Antigen detection in experimentally infected foxes

Antigen was detected between 5 and 11 weeks post-inoculation (wpi) in foxes in both experiments (Figs 2 and 3). Seven animals, four in the first and three in the second trial, with WB equal to 1, 2, 14, 15, 16

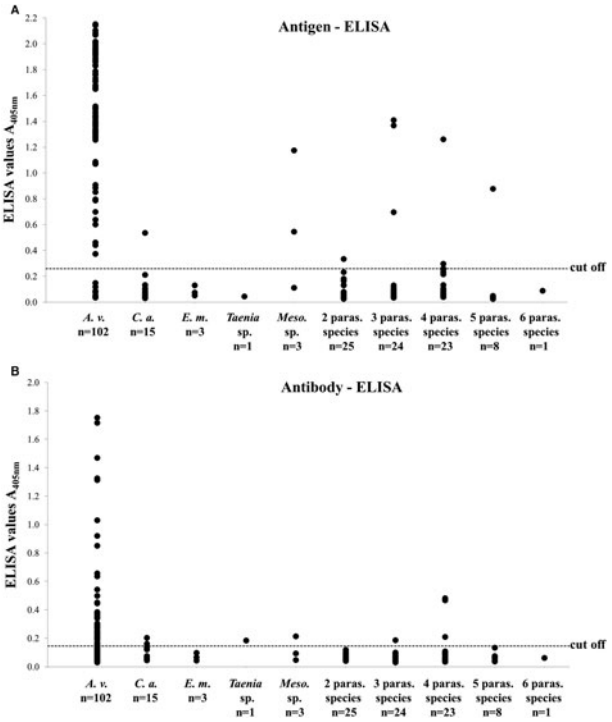

Fig. 1. Evaluation for cross-reactions of the ELISAs for detection of circulating *Angiostrongylus vasorum* (*A.v.*) antigen (A) and specific antibodies (B) in fox blood samples from 102 foxes harbouring *A. vasorum* and 103 wild foxes naturally infected with only *Capillaria aerophila* (*C.a.*, syn. *Eucoleus aerophilus*), *Toxocara canis* (*T.c.*), *Echinococcus multilocularis* (*E.m.*), *Taenia* sp., *Mesocestoides* sp., other nematodes or multiple parasitic infections. Columns stating two to six parasite species do not include foxes infected with *Angiostrongylus vasorum*. Foxes above cut-off and infected with more than two parasite species (other than *A. vasorum*) were infected with: *C. aerophila, Crenosoma vulpis, T. canis, E. multilocularis, Taenia* sp., *Mesocestoides* sp. and/or other nematodes.

(2*x*) and 36, respectively, did not become antigen positive. One fox of trial 2 (group A) that died shortly after inoculation (not related to the *A. vasorum* infection) and before the second blood draw was not included in the serological follow-up.

Six of 28 foxes (21·4%) in trial 1 and eight of 47 foxes (17%) in trial 2, which were first positive at 5 wpi, showed a decrease in antigen values at 6 or 7 wpi; eight of them (two of the first and six of the second experiment) actually dropped under the cut-off value. However, after this drop, antigen values increased again in all foxes that were followed up for a longer period of time (and not euthanized at 9 wpi). By 10 wpi, all foxes were seropositive again. The other 61 foxes, which did not show this drop, had a steady increase in antigen OD values for several weeks and kept steady circulating antigen levels until nine or 22 wpi, respectively (Figs 2 and 3). The six non-inoculated control foxes of the first experiment were always seronegative.

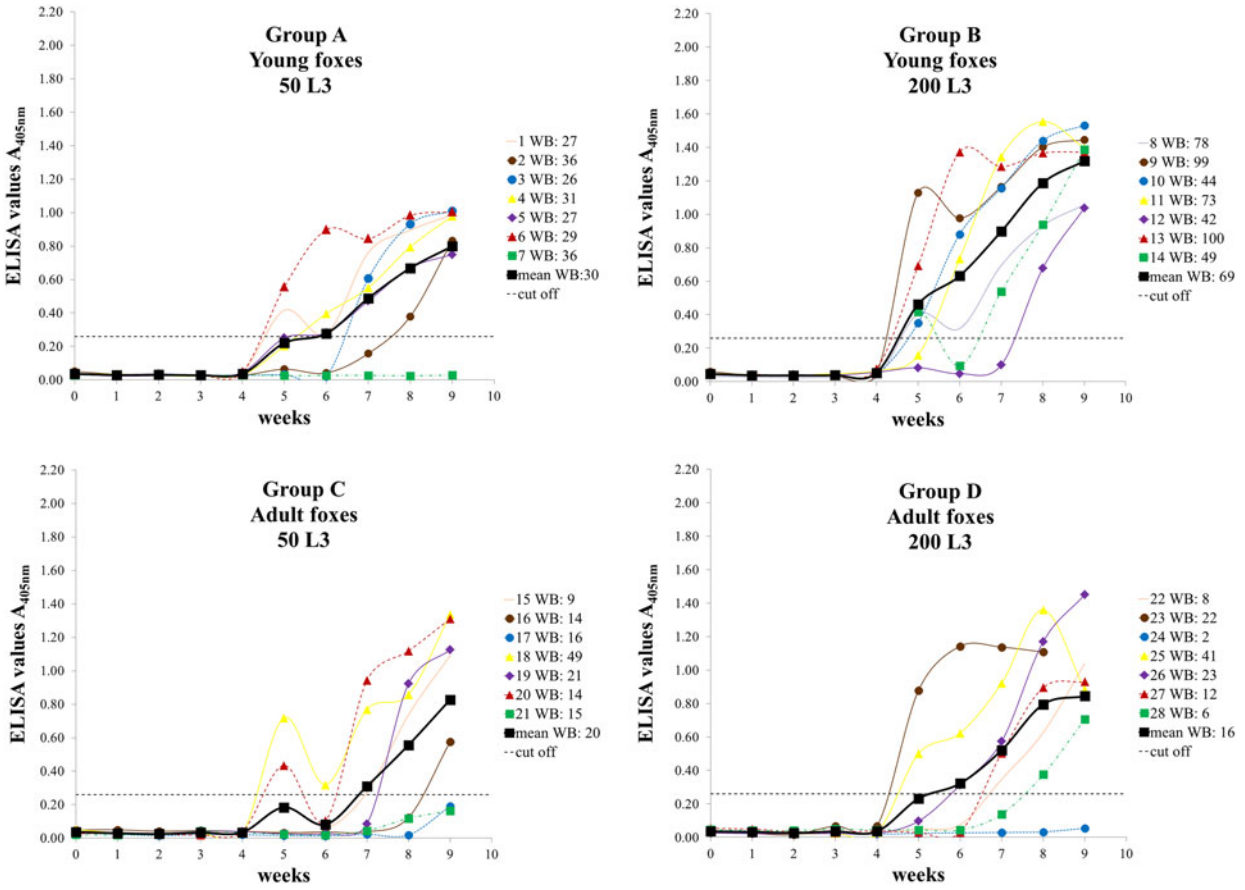

Fig. 2. Detection of circulating antigen of *Angiostrongylus vasorum* (trial 1): Weekly antigen OD values of individual foxes belonging to four different inoculation groups, and mean OD values of each group. Fourteen young foxes (groups A and B) and 14 adult foxes (groups C and D), seven of each age group, were inoculated with 50 or 200 *A. vasorum* third-stage larvae (L3). Blood was drawn once weekly until 9 wpi, when animals were euthanized and worm burden (WB) was determined.

Comparing the groups of trial 1, five (71·4%) out of seven of the young foxes inoculated with 200 L3 (group B) were already seropositive at 5 wpi. In contrast, only two (28·6%) foxes were seropositive at 5 wpi from the juvenile group inoculated with 50 L3 (group A) as well as from both adult groups (C and D, Fig. 2).

Foxes of groups A, B and D of trial 2 showed comparable dynamic patterns of measured antigen values over time. At 5 wpi, nine foxes (45%) of group A were found antigen positive, while four (57·1%) foxes were seropositive in group B and six foxes in group D (85·7%). The last fox to become antigen positive did at 11 wpi. 10–12 wpi all groups reached a plateau. The two infected control groups C and E showed antigen levels comparable to group A (Supplementary Fig. S3).

Antibody detection in wild foxes

Suitability of old fox blood samples (including full blood samples and tissue fluid) for detection of antibodies was confirmed by *T. canis* positive results among the 65 reference wild red foxes (from the USA and Switzerland), and in the *A. vasorum* positive control dogs, whereas the samples from SPF dogs were negative.

Forty-three of 102 foxes (46·1%) with *A. vasorum* adults at necropsy, and nine of 113 (8·0%) foxes negative at necropsy, were antibody positive, four (3·5%) of which were also antigen positive (Table 1). A sensitivity of 42·2% and a specificity of 92·0% were calculated based on necropsy findings.

Eight of 103 surveyed foxes (7·8%) negative for *A. vasorum* at necropsy but infected with other parasites had antibody values above cut-off, two infected with *C. aerophila*, one with *Taenia* sp. and one with *Mesocestoides* sp., and four with multiple endoparasite species (excluding *A. vasorum*) (Fig. 1). Three foxes were positive in both, the Ag-ELISA and Ab-ELISA, one fox was infected with *C. aerophila*, another with *Mesocestoides* sp., and the last one with four helminth species other than *A. vasorum*.

When combining the two ELISAs, the 'OR' combination resulted in a combined sensitivity of 94·4% and a specificity of 82·3%. With the 'AND' combination, sensitivity was 38·4% and specificity was 99·2%.

Antibody detection in experimentally infected foxes

Detailed trends for antibody detection are displayed in Figs 4 and 5. The most noticeable increase in antibody OD values was observed between 5 and 7 wpi

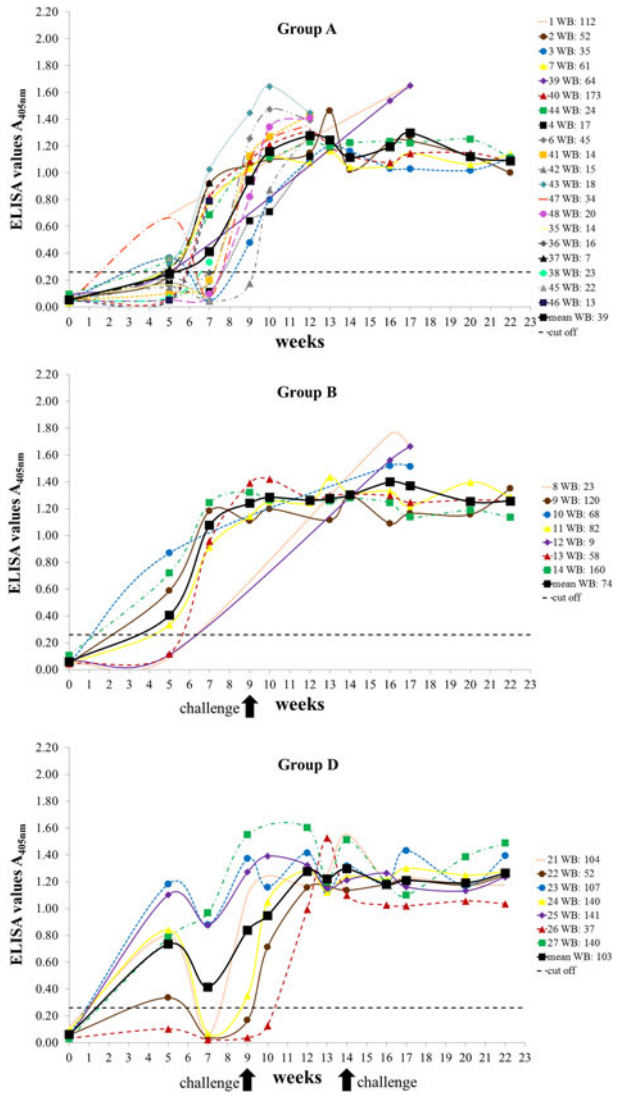

Fig. 3. Detection of circulating antigen of *Angiostrongylus vasorum* (trial 2): OD values of foxes of groups A [$n = 20$; inoculated with 100 third-stage larvae (L3)], B ($n = 7$; inoculated twice with 100 L3) and D ($n = 7$; inoculated three times with 100 L3) as well as group arithmetic means are shown starting from inoculation until necropsy, when worm burden (WB) was determined. Blood sampling is indicated with a marker for each individual. Inoculation challenges are indicated with an arrow.

for the foxes of both experiments. Seventeen (60·7%) foxes of trials 1 and 23 (48·9%) foxes of trial 2 had a peak at 6 or 7 wpi, respectively, followed by a decrease in values over the next couple of weeks. Six challenged foxes further showed noticeable increase in antibody OD values 3–4 or 7 weeks after being challenged.

In trial 1 (Fig. 4), follow-up antibody values of the juvenile foxes (both with high and low inoculation dose) and the adult foxes infected with 50 L3 were comparable. Throughout the experiment the values of the adult foxes infected with 200 L3 (group D) were less uniform and variable over time for each animal. Adult foxes had higher OD values by trend when compared with juvenile foxes.

In trial 2 (Fig. 5), antibody values of foxes in group A (inoculated once) were highly variable after the initial increase about 7 wpi, with foxes showing then increasing or decreasing levels, or showing both, up to 22 wpi. In group B (inoculated twice, initially and 9 wpi), antibody levels had a decreasing tendency from 7 wpi after initial inoculation until approximately 7 weeks after challenge, where antibody levels had increased in four of seven foxes. Comparable trends were observed in group D (inoculated three times): antibody levels decreased from 7 wpi and increased again 7 weeks after the first challenge in six foxes. At 6 or 8 weeks post second challenge all seven foxes had a minor increase in antibody OD values. The infected control groups C and E showed antibody values comparable with group A (Supplementary Fig. S4).

One fox of the first and two foxes of the second trial dropped below the cut-off after peaking at 5 and 7 wpi, respectively. Of these, one remained seronegative for 9 weeks before becoming positive again at 20 wpi. In general, after 7 wpi the antibody values of individual foxes were highly diverse. The six uninfected control foxes of the first experiment remained antibody seronegative.

Correlations between ELISA OD values and WBs and larvae per gram of feces

In experimentally infected foxes, both WB and mean LPG had weak Pearson correlations ($r = 0{\cdot}412$, $P \leqslant 0{\cdot}0001$, $r^2 = 0{\cdot}169$; and $r = 0{\cdot}585$, $P \leqslant 0{\cdot}0001$, $r^2 = 0{\cdot}342$, respectively) to antigen OD values at necropsy. Antibody values did not correlate with either WB or LPG ($r = -0{\cdot}033$, $P = 0{\cdot}786$, $r^2 = 0{\cdot}001$; and $r = 0{\cdot}078$, $P = 0{\cdot}108$, $r^2 = 0{\cdot}006$, respectively) (Fig. 6). Single foxes showed high antibody OD values and low WB, while others had low antibody OD values with high WB (Figs 4 and 5). As an example, two foxes in trial 2 of the same infection group (D) harbouring the exact same high number of adult *A. vasorum* specimens (WB: 140) had highly contrasting antibody OD trends (Fig. 5).

In the wild Swiss foxes (Fig. 7), WB had weak correlations to both antigen and antibody OD values: $r = 0{\cdot}569$, $P \leqslant 0{\cdot}0001$, $r^2 = 0{\cdot}323$, and $r = 0{\cdot}378$, $P \leqslant 0{\cdot}0001$, $r^2 = 0{\cdot}143$, respectively.

DISCUSSION

This study shows that *A. vasorum* antigen and antibody detection in fox sera and whole-blood samples is possible with the previously described ELISAs (Schnyder *et al.* 2011; Schucan *et al.* 2012) and therefore represent potential suitable methods for epidemiological screening of wild foxes for *A. vasorum*. Whereas the Ag-ELISA has a high sensitivity and a high specificity with blood of naturally

Table 1. Contingency table of the results of the ELISA for detection of circulating *Angiostrongylus vasorum* antigen and the ELISA for detection of specific antibodies based on 215 necropsied Swiss red foxes between the years 2013 and 2015

	Necropsy		
Serology	Positive *n* (%)	Negative *n* (%)	Total *n* (%)
Antigen and antibody positive	41 (40·2)	4 (3·5)	45 (100)
Antigen positive and antibody negative	52 (51·0)	8 (7·1)	60 (100)
Antigen negative and antibody positive	2 (2·0)	5 (4·4)	7 (100)
Antigen and antibody negative	7 (6·9)	96 (85·0)	103
Total	102	113	215

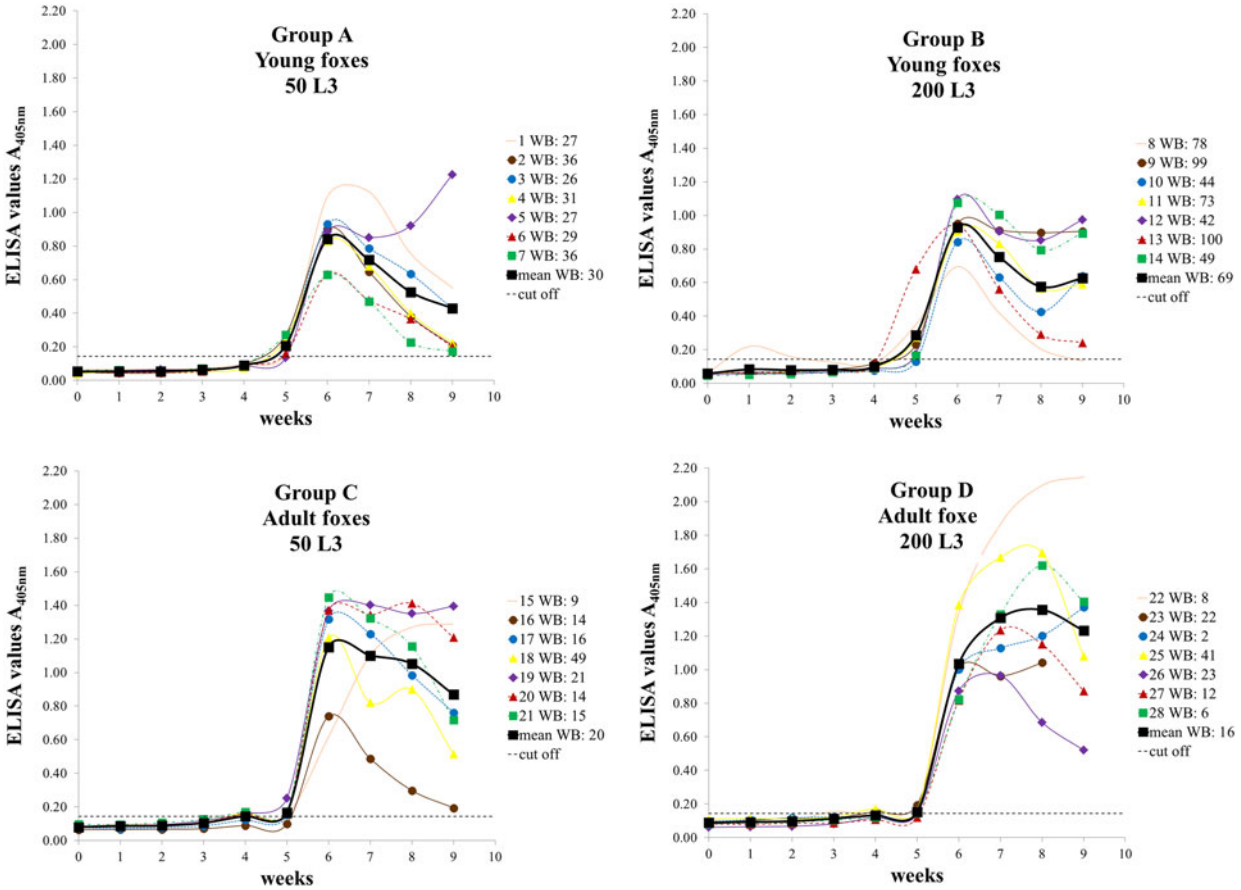

Fig. 4. Detection of specific antibodies against *Angiostrongylus vasorum* (trial 1): antibody OD values of individual foxes belonging to four different inoculation groups and mean OD values of each group. Fourteen young foxes (groups A and B) and 14 adult foxes (groups C and D), seven of each age group, were inoculated with 50 or 200 *A. vasorum* third stage larvae (L3). Blood was drawn once weekly until 9 wpi, when animals were euthanized and worm burden (WB) was determined.

infected foxes, the specificity of the Ab-ELISA was comparably high, but sensitivity was low.

For the past years, the Ag-ELISA and Ab-ELISAs have successfully been used to detect *A. vasorum* infections in dogs (Guardone *et al.* 2013; Schnyder *et al.* 2013*a*, *b*; Lurati *et al.* 2015). In dogs, a comparison between these serological methods, the Baermann technique and PCR performed with blood, feces and tracheal swabs showed that the most consistent results were obtained in ELISAs (Schnyder *et al.* 2015). The Ag-ELISA can detect positive dogs as early as 5 wpi and shows a sensitivity of 95·7% and a specificity of 94% (Schnyder *et al.* 2011) for dog samples. These results are comparable with the results we obtained with the wild fox samples, where sensitivity was 91·2% and specificity 89·4%, and antigen detection is possible after 5–7 wpi. As in dogs, antigen detection in foxes is consistent throughout the course of infection over a long period of time. In both trials antigen values of foxes remained high after 6–10 wpi in foxes receiving single or repeated

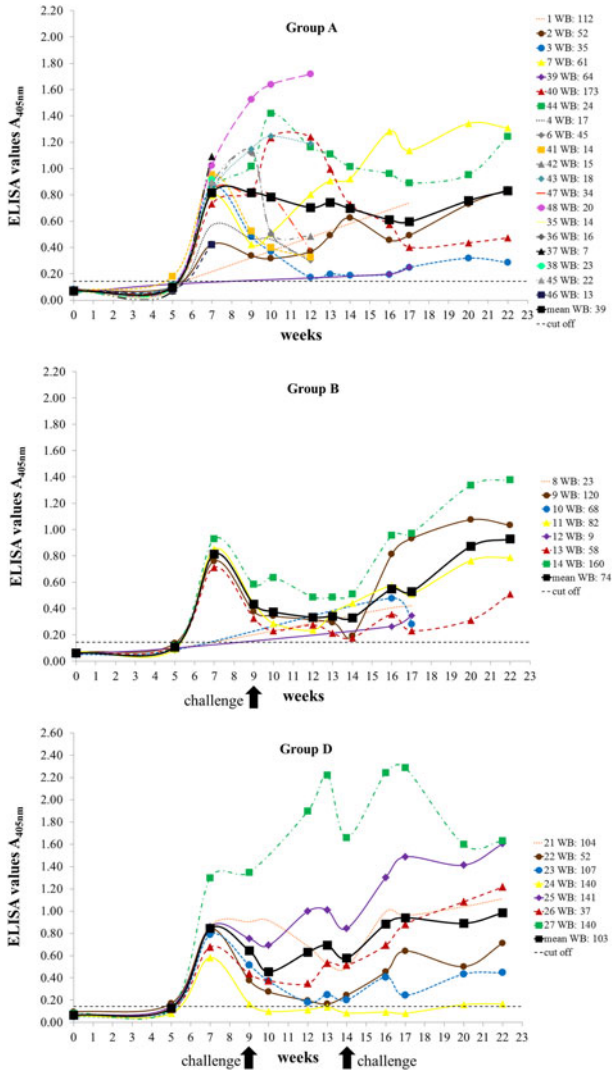

Fig. 5. Detection of specific antibodies against *Angiostrongylus vasorum* (trial 2): OD values of foxes of groups A [$n = 20$; inoculated with 100 third-stage larvae (L3)], B ($n = 7$; inoculated twice with 100 L3) and D ($n = 7$; inoculated three times with 100 L3) as well as group arithmetic means from inoculation until necropsy, when worm burden (WB) was determined. Blood sampling is indicated with a marker for each individual. Inoculation challenges are indicated with an arrow.

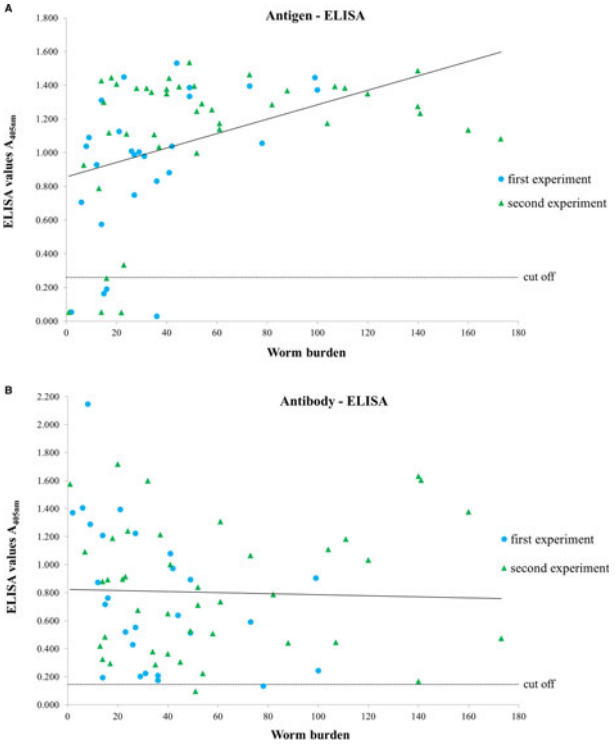

Fig. 6. Scattering of *Angiostrongylus vasorum* worm burden against antigen (A) ($r = 0{\cdot}412$, $P \leqslant 0{\cdot}0001$, $r^2 = 0{\cdot}169$) or antibody (B) ($r = -0{\cdot}033$, $P = 0{\cdot}786$, $r^2 = 0{\cdot}001$) OD values determined at necropsy of experimentally inoculated foxes (trial 1, $n = 27$, blue circles, trial 2, $n = 42$, green triangles).

inoculations. The results of the eight foxes, which persisted antigen negative and the two foxes, which had their first positive antigen values 10–11 wpi, indicate that antigen may not always be detected or may only be detected later during infection. If these findings are associated with the nine naturally infected foxes (each harbouring only one worm), which were negative in the Ag-ELISA, one may assume that these wild foxes were shot about 4 weeks after ingestion of L1 [first adult stages are present 25 days after inoculation (Guilhon and Cens, 1973)] and had no time to build-up sufficient circulating antigen levels yet, or simply that one adult *A. vasorum* specimen is not sufficient to produce antigen levels above the cut-off.

A positive correlation between WB and antigen OD values was demonstrated: the presence of a higher number of adult specimens induced higher levels of circulating antigen and therefore higher antigen OD values. Thus, higher values in foxes may correlate with higher infection burdens. Similarly, there was a positive correlation of LPG at necropsy and antigen values. Higher mean numbers of L1 were observed in foxes of groups that were challenged, and these were also harbouring higher mean numbers of adult specimens (Woolsey *et al*. 2017). Nevertheless, LPG findings from foxes throughout the course of infection were not consistent; some did not even shed L1 at all (Webster *et al*. 2017). Intermittent excretion (Oliveira-Júnior *et al*. 2006) of larvae needs to be taken into account, and larval output is not a stable indicator for higher infection rate (Webster *et al*. 2017). On the other hand, antigen values proved to be very stable during the course of the infection and represent a more reliable indicator for WB. The reasons for the decrease in antigen values after 5 wpi, which was seen in 14 out of 75 (18·7%) foxes, are not fully understood. During this time, ingested L3 reach the adult mature stage in the definitive host and females carry eggs, which are then shed into the arteries. Six to seven wpi infected animals start shedding L1 (Guilhon and Cens,

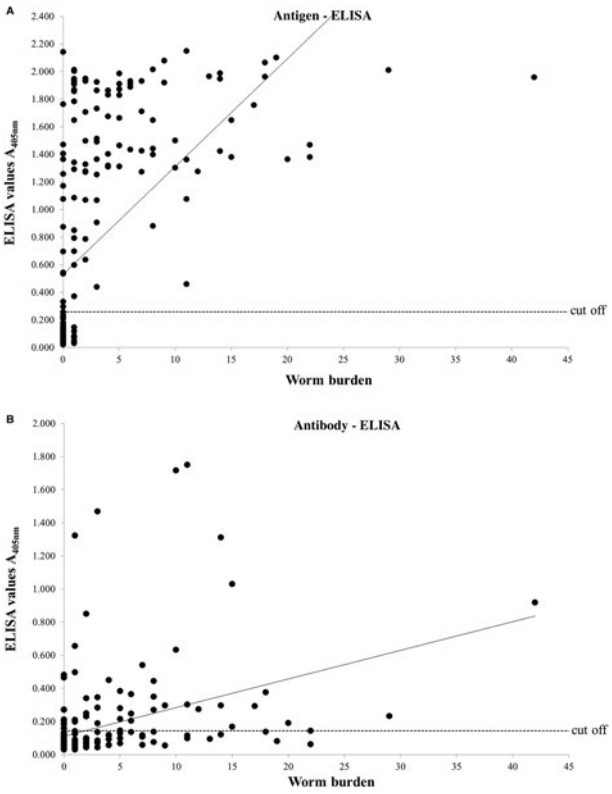

Fig. 7. Scattering of *Angiostrongylus vasorum* worm burden against antigen (A) ($r = 0{\cdot}569$, $P \leqslant 0{\cdot}0001$, $r^2 = 0{\cdot}3234$) or antibody (B) ($r = 0{\cdot}378$, $P \leqslant 0{\cdot}0001$, $r^2 = 0{\cdot}1429$) OD values determined at necropsy of 215 wild Swiss foxes.

1973); however, some variability due to different strains and host-switching between foxes and dogs could be hypothesized. Different lineages of *A. vasorum* have been described (Jefferies *et al*. 2009), but no evident differences of biological aspects or virulence are, to our knowledge, mentioned. Considering therefore the life cycle of *A. vasorum*, there seems to be no reason for a decrease in circulating antigens, as it is supposedly produced by adult worms; but interestingly, also about 6–7 wpi, a peak in antibody values (which is afterwards followed by a decrease) was observed. Antigen–antibody complex formation leading to a clearance of both may explain this phenomenon. Indeed, such complex formation has been discussed in *D. immitis* infections in dogs as well as in cats (Little *et al*. 2014; Drake *et al*. 2015), leading to reduced antigen detection. In previous studies, a relevant role for immunopathogenesis in the lungs of *A. vasorum* infected dogs was attributed to the formation of complexes of immunoglobulins, fibrin and complement, with predominant IgA (Caruso and Prestwood, 1988). The formation of antigen–antibody complexes were therefore also proposed as a causal mechanism underlying behind some antigen negative *A. vasorum* infected dogs seroconverting to positive after heat treatment (Schnyder *et al*. 2014).

The sandwich Ab-ELISA with somatic antigen and purified with mAb 5/5 can detect infections in dogs 3–5 wpi, with a sensitivity of 81% in naturally infected dogs and a specificity of 98·8% in randomly selected dog samples (Schucan *et al*. 2012). In contrast, the same ELISA setting has a sensitivity of 42·2% when performed with wild fox samples. In experimentally infected foxes, antibody detection was possible in 38·7% of animals 5 wpi and in 100% 7 wpi. A noticeable difference between dogs and foxes is seen 6–7 wpi. If dogs are left untreated, specific antibodies are detectable for several months. In contrast, the present study demonstrates decreasing antibody levels in more than half of all foxes after 7 wpi (in a few foxes below cut-off), although animals never received anthelmintic treatment and the presence of adult specimens was proven at necropsy.

The low sensitivity of the Ab-ELISA in naturally infected foxes can be explained with this OD value drop followed by a continuous trend of decreasing OD values over time, occurring in more than 50% of experimentally infected foxes. Assuming persistency of this trend, OD values can therefore drop under the cut-off level, if foxes are not reinfected. In fact, in the experimentally infected foxes we see a trend towards increasing antibody values after challenges, and higher infection doses leading to higher antibody values in both juvenile and adult foxes. Simultaneously, WB and antibody values at necropsy did not correlate in experimentally infected foxes, nor did inoculation dose and WB in adults (Webster *et al*. 2017). Antibody values in the experimentally infected farm foxes were highly variable over time following comparable inoculation doses, within groups and even within individuals. At once, the naturally infected foxes originated from different areas and environments; from some no parasites were isolated and others were infected with up to six different parasite species. Therefore, naturally infected foxes were presumably exposed to highly varying parasitic and/or other immunological stimuli, and antibody response in naturally infected foxes may be even more diverse than in experimentally infected foxes.

In opposition to low sensitivity, specificity of the Ab-ELISA with fox samples was high (92·0%). Interestingly, in our study 10·6% of foxes negative at necropsy were antigen seropositive and 8·0% of necropsy negative foxes were antibody seropositive. A total of 3·5% ($n = 4$) of foxes, which were negative at necropsy were positive in both ELISAs. Likely though, these results indicate that approximately 4–11% of *A. vasorum* positive foxes had probably been missed upon dissection, supporting that the dissection technique is not 100% accurate (Houpin *et al*. 2016). In fact, it is not possible to open every small vessel in the lung to recover all parasites, which are thin and relatively small (particularly males). The dissection technique, which was used to

examine naturally infected foxes, is comparable with the one described by Houpin *et al.* (2016). These authors compared dissection with PCR of BALF (bronchoalveolar lavage fluid) and with a serological in-clinic assay (Angio Detect™, IDEXX Laboratories, Westbrook, Maine, USA) for detection of *A. vasorum* antigen in tissue fluid, obtaining a sensitivity of 84·1, 69·5 and 76·8%, respectively, assuming positivity for all samples positive by any of the methods applied. The in-clinic commercial ELISA detects circulating antigen as well, however, as shown for dogs, with a lower sensitivity than the ELISA employed here (Schnyder *et al.* 2014). Comparably, this latter ELISA showed a higher sensitivity (91·2%) than the in-clinic assay using fox material (76·8%, Houpin *et al.* 2016).

Dissection may lead to false-negative results, especially when the animals are infected with few parasites or were only recently infected, and therefore this method cannot be considered as a gold standard. Likewise, at least the three foxes with apparent cross-reactions being positive for both antigen and antibody detection (and positive for *C. aerophila, Mesocestoides* sp. or three parasite species, respectively) but negative for *A. vasorum* at dissection, and possibly also other apparently cross-reacting sera, with high probability had adult worms that were not detected. Conducting cross-reactivity studies with foxes experimentally infected with specific helminths other than *A. vasorum* could have helped supporting specificity results; such samples were however not available. Complementary to necropsy, another approach to better estimate specificity and sensitivity of the present ELISAs may consist in Bayesian statistical inference techniques, as formerly described for evaluating different methods to identify cows infected with *Taenia saginata* in absence of a gold standard test (Eichenberger *et al.* 2013).

In previous studies with experimentally infected foxes, the animals had only mild haematological changes when infected and challenged with *A. vasorum* L3 (Woolsey *et al.* 2017). Merely the number of eosinophils, basophils and, in some groups, lymphocytes slightly increased at certain time points in some of the infection groups (Woolsey *et al.* 2017). The amount of neutrophils did not change, which suggests a very mild or even a lack of inflammatory haematological reaction. Lymphocyte numbers are relevant in this context because B cells are responsible for the adaptive part of humoral immunity as antibody producers. The antibody responses observed in the present study, though, are merely a reaction to a single specific epitope. The haematological changes, a 7-week interval of antibody increase after each infection or challenge, combined with the finding of increasing mean WB in challenged groups, speak for the absence of protective immunity in foxes. Foxes can apparently tolerate a patent *A. vasorum* infection over a long period of time, suggesting a balanced host–parasite interplay between foxes and *A. vasorum* that is particularly well adapted to favour the transmission of the parasite (Webster *et al.* 2017; Woolsey *et al.* 2017).

Current knowledge on *A. vasorum* infections or prevalence in foxes mostly relies on necropsy results. However, the procedures for isolation of *A. vasorum* from dead foxes can be time consuming and laborious. The presented ELISAs may represent an alternative and efficient approach for mass-screening of *A. vasorum* infections in fox populations, also when lungs and hearts are not available. Combining both ELISAs, if the 'OR' combination is applied, sensitivity can be further increased, reaching at least 94·4%. In addition, the combination of both ELISAs may provide valuable indications of the time of infection or reinfection in foxes: animals which are only positive in the Ag-ELISA, but negative in the Ab-ELISA most likely were infected weeks or months ago and were not reinfected during this time, while foxes only seropositive for antibodies may have been infected 5–7 weeks before.

Our findings from experimentally and naturally infected foxes provide some evidence that foxes develop parasite tolerance and confirm the potential for life-lasting survival of *A. vasorum* in definitive hosts (Rosen *et al.* 1970). This contributes to our understanding of the epidemiology of *A. vasorum* infection in red foxes, underpinning their significance for the establishment of endemic foci of the parasite and for the epidemiological dynamic that is evident in many parts of Europe where *A. vasorum* has been found over the last few decades.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017000427

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SUPPLEMENTARY FIGURES

Figure S1: Experimental design of the first experiment, trial 1 (see also Webster *et al.* (2017)): Fourteen adult and 14 young foxes were inoculated with either 50 or 200 *Angiostrongylus vasorum* third stage larvae (L3). Three young and three adult non-inoculated foxes were used as controls. Blood draw was conducted weekly and faecal samples were collected and analysed three times a week starting 4 weeks post inoculation (wpi). All animals were euthanized and examined 9 wpi.

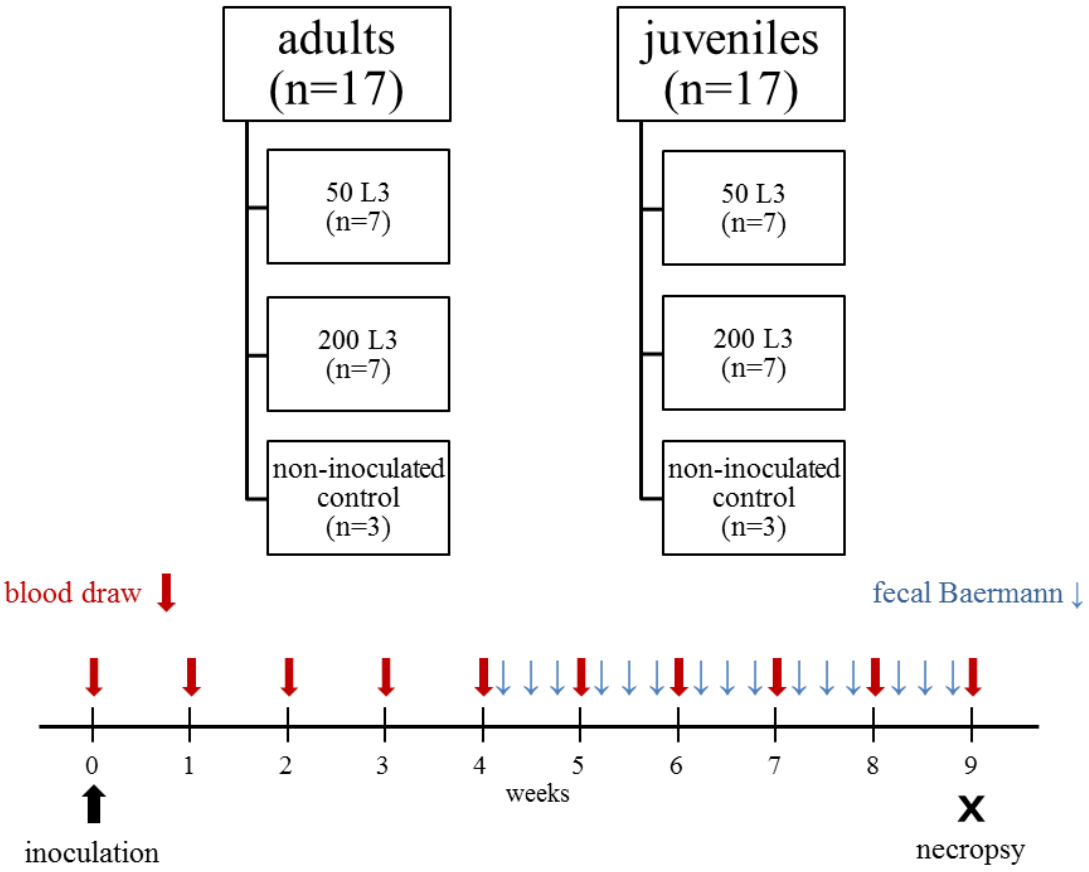


Figure S2: Experimental design of the second experiment, trial 2 (see also Woolsey *et al*. (in press)). Five groups of 21, 7, 7, 7 and 6 foxes, respectively, were inoculated with 100 *Angiostrongylus vasorum* third stage larvae once (group A), twice (group B) or three times (group D) and necropsied at different time points. Groups C and E acted as infectivity control groups. Black arrows: inoculation or challenge; crosses: necropsy; red arrows: blood draws.

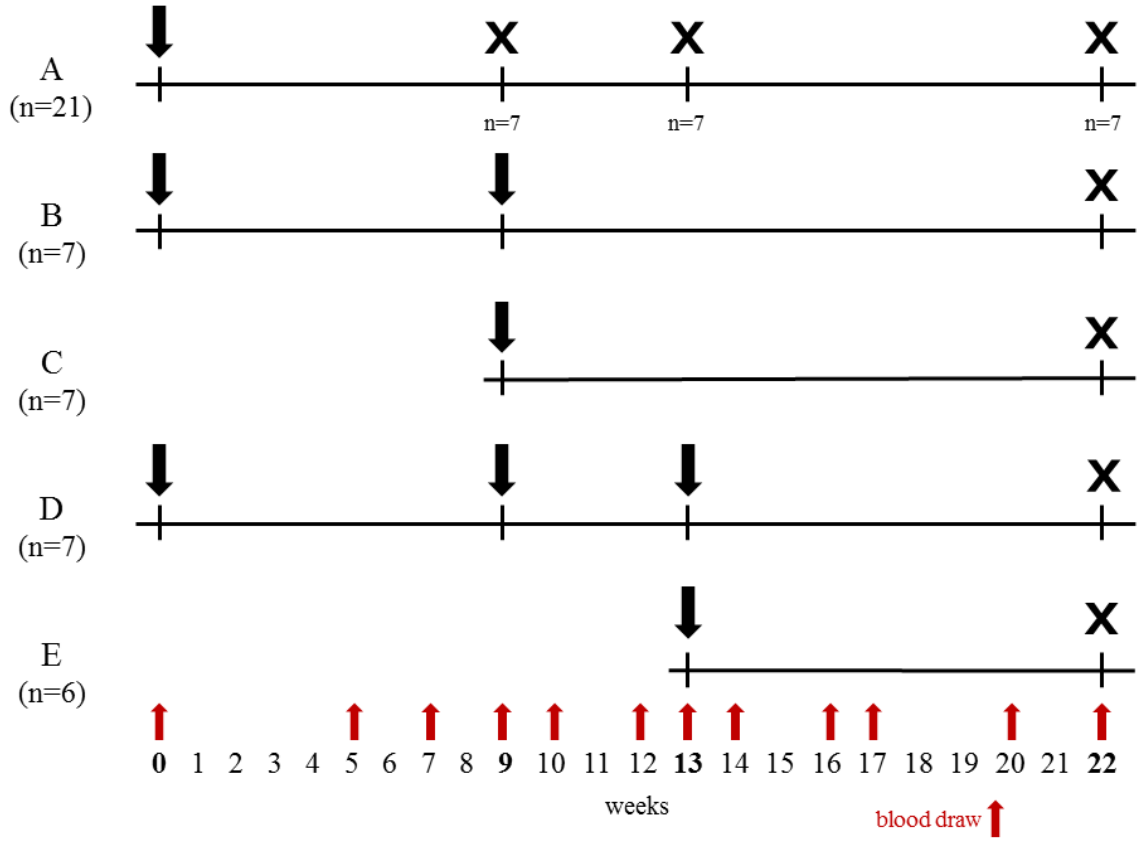
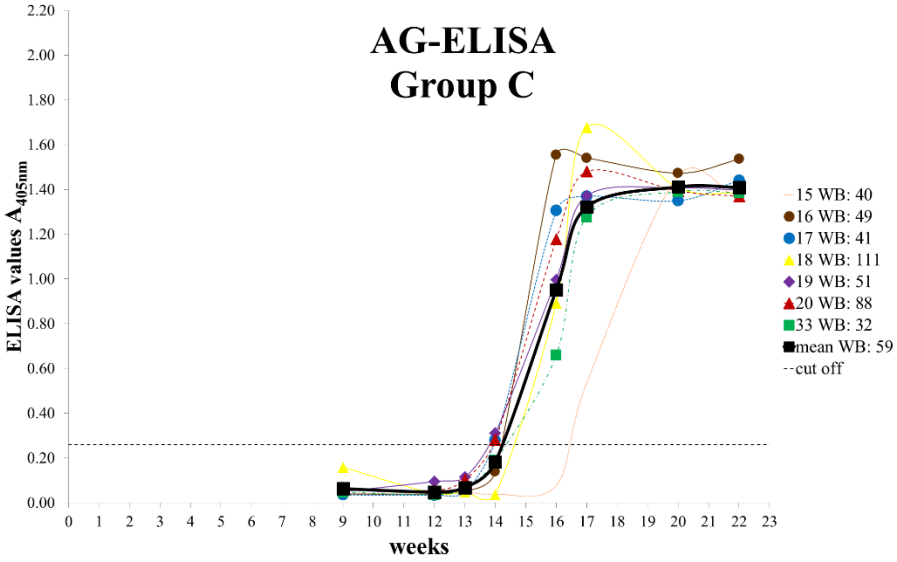


Figure S3: Trial 2, control groups: detection of circulating antigen of *Angiostrongylus vasorum*: OD values of foxes of groups C (n=7; inoculated with 100 L3) and E (n=6; inoculated with 100 L3) as well as arithmetic mean OD values from inoculation until necropsy, when worm burden (WB) was determined. Blood withdrawals are indicated with a marker for each individual.



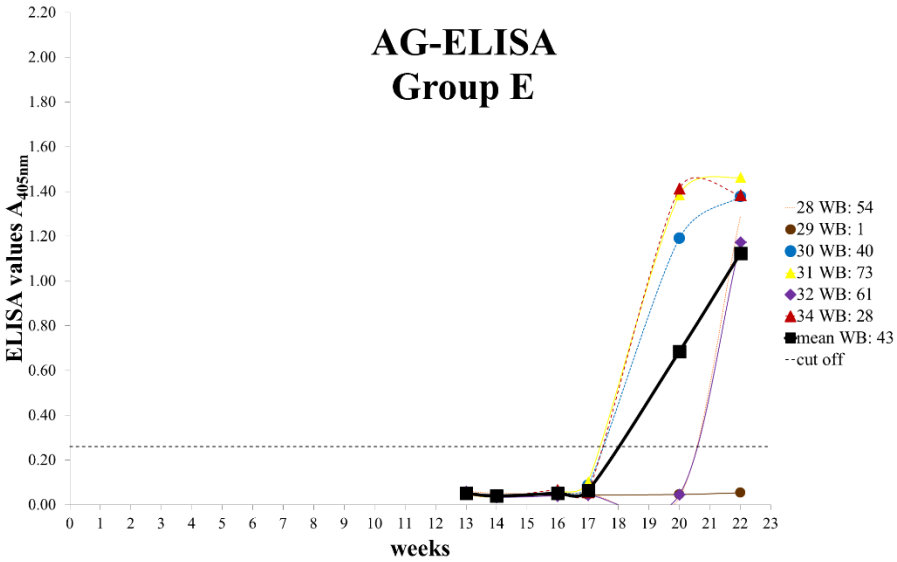
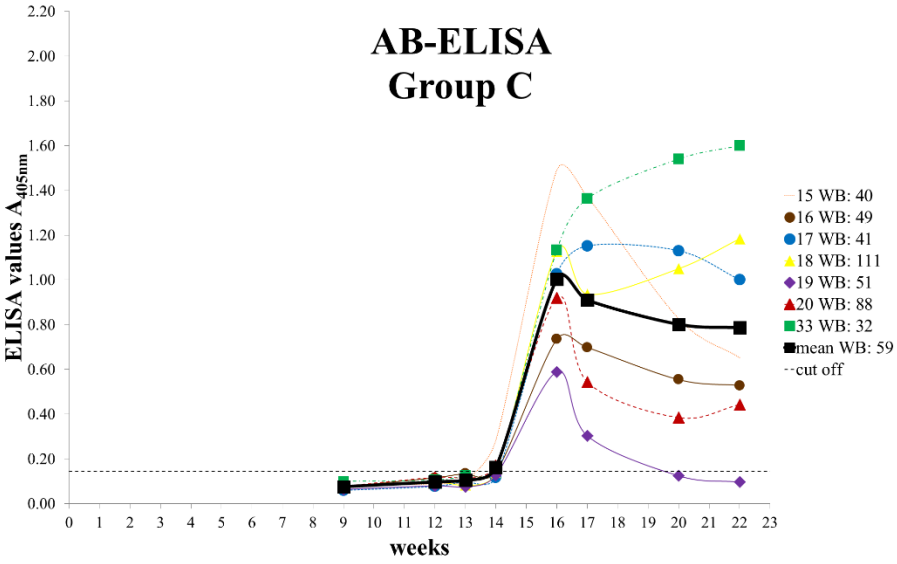
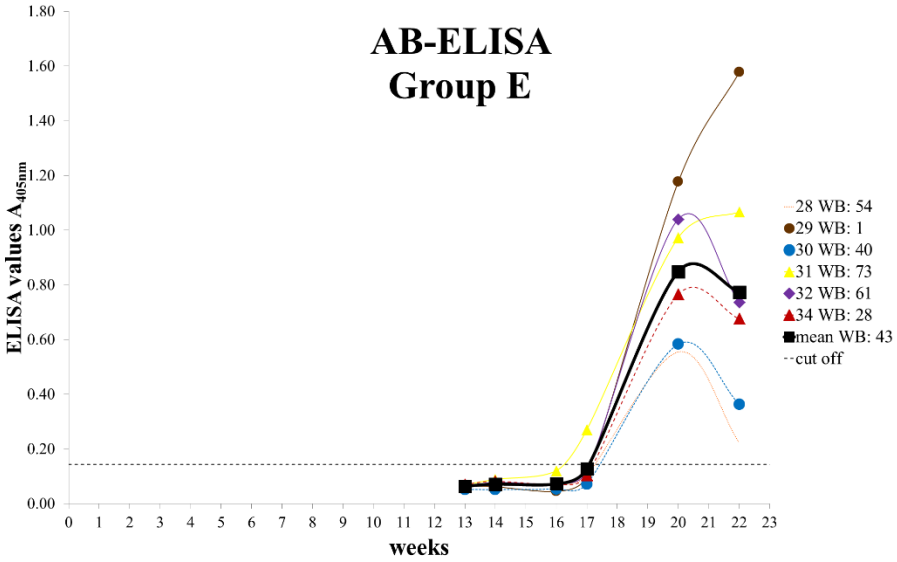


Figure S4: Trial 2, control groups: detection of specific antibodies against *Angiostrongylus vasorum*: OD values of foxes of groups C (n=7; inoculated with 100 L3) and E (n=6; inoculated with 100 L3) as well as arithmetic mean OD values from inoculation until necropsy, when worm burden (WB) was determined. Blood withdrawals are indicated with a marker for each individual.





Impact of heat treatment on antigen detection in sera of *Angiostronylus vasorum* infected dogs

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Abstract

The diagnosis of angiostrongylosis is usually confirmed by faecal analysis with detection of first stage larvae. Recently, serological tests for detection of circulating *A. vasorum* antigen or specific antibodies have been adopted for individual diagnosis and epidemiological studies. However, although confirmed positive at necropsy, antigen detection was not possible in single experimentally as well as naturally infected dogs, possibly due to immune complex formation. The aim of this study was to evaluate the effect of heat treatment on detection of *A. vasorum* antigen in sera of experimentally (n=21, 119 follow-up sera) and naturally (n=18) infected animals. In addition, sera of dogs showing clinical signs compatible with angiostrongylosis (n=10) and of randomly selected dog sera samples (n=58) were evaluated. Sera were subject to heat treatment at 100 °C after addition of 0.5M EDTA (dilution 1:5) and tested with ELISAs for detection of circulating *A. vasorum* antigen before and after heat treatment. Untreated samples of experimentally infected dogs started to be positive three weeks post inoculation (wpi). Between five and eleven wpi the percentage of positive samples increased over time from 33.3% to 90%. Single dogs were still negative between 12 and 15 wpi. Overall, between five and fifteen wpi, 50.6% (45/89) of the available samples were seropositive. By EDTA/heat treatment five previously positive samples collected before six wpi became negative. Instead, of the ones collected after five wpi, 68.5% (61/89) were antigen positive. Between seven and ten wpi additional 34.6% (18/52) became positive compared with untreated samples. Thirteen of 18 naturally infected dogs were antigen positive before and 15 after EDTA/heat treatment, respectively. Untreated samples of three dogs with suspect clinical signs were antigen positive, of which only one (already strongly positive before treatment) remained positive after EDTA/heat treatment, allowing better discrimination between positive and negative samples. One of 58 random samples was antigen positive prior to treatment. After EDTA/heat treatment, this sample became negative and another turned positive. Furthermore, 74 samples were tested with the in clinic assay Angio Detect™ before and after EDTA/heat treatment, after which one additional sample of an experimentally infected dog collected eight wpi became positive. In conclusion, heat treatment can improve *A. vasorum* antigen detection between seven to ten wpi by immune complex disruption, but may impair the detection beforehand.

Key words: *Angiostrongylus vasorum*, dog, serum, heat treatment, ELISA, antigen, serology

Background

Angiostrongylus vasorum has become a frequently diagnosed parasite in dogs in many European countries over the last decades. Due to the manifestation of severe clinical signs, a reliable and efficient method for diagnosing the infection is essential. The frequently used copromicroscopic method, the Baermann-Wetzel technique [1] detecting first stage larvae (L1), is recently complemented by other techniques, such as Enzyme-Linked Immunosorbent Assays (ELISAs) [2, 3] and biomolecular methods [4], as well as by a rapid in-clinic assay (Angio Detect^{TM} Test, IDEXX Laboratories, Westbrook, Maine, USA). The ELISA for detection of circulating *A. vasorum* antigen and the ELISA for detection of specific antibodies purified with monoclonal antibodies give consistent results over the duration of infection [2, 3, 5]. Antigen can be detected as early as 35 days post inoculation, however in some dogs antigen is detected later or, in single cases, not detected at all, although such dogs were shown harboring up to 165 adult parasites [2]. Similar difficulties have been reported for other serological tests detecting parasitic antigen, e.g. in the case of *Dirofilaria immitis* in cats [6]. Little et al. [7] have recently reported that heat treatment of sera improves the detection of *D. immitis* antigen in infected cats. The same treatment method was also effectively used for sera of *D. immitis* infected dogs [8, 9]. Comparable heat treatment methods for sensitivity improvement have additionally been reported in the past for sera containing antigens of other pathogens such as *Histoplasma* [10], *Coccidioides* [11], *Aspergillus* [12], *Candida albicans* [13] and human immunodeficiency virus type 1 [14]. Apart from heat treatment, acid dissociation is another method described to improve antigen detection [15-17]. Heat treatment and acid dissociation are both believed to disrupt immune complexes such as antigen – antibody complexes and therefore make antigen accessible again for detection by ELISA [18]. Antigen - antibody complexes were described to occur in infections with different pathogens in dogs, such as with ehrlichiosis [19] or leishmaniosis [17]. They may form if antigen and antibodies are both circulating in a high concentration, masking therefore an infection [20]. Reports for immune complex formation in dogs infected with *A. vasorum* and their pathogenic effect are scant [21]. The aim of this study was to evaluate the effect of heat treatment of sera on antigen detection by ELISA and by the rapid in-clinic assay in dogs infected with *A. vasorum*.

Methods

Source of sera samples

A total of 205 dog sera were evaluated.

a) One-hundred-and-nineteen sera samples originated from 21 dogs experimentally infected with *A. vasorum* from previously performed studies [22-24] before and at various stages of infection. From eight dogs weekly samples were available starting before or shortly after inoculation until necropsy, from the other 13 dogs a selected number of sera samples were available. Worm burdens were determined at necropsy (varying between 1-170). Experimental trials were conducted according to the Swiss guidelines for animal experimentation and approved by the Cantonal Veterinary office.
b) Eighteen sera samples originated from dogs naturally infected with *A. vasorum*, presented between the years 2005 and 2017 and confirmed positive with either the Baermann technique or at necropsy.
c) Further ten samples were obtained from dogs suspect for angiostrongylosis showing clinical signs which included one or several of the following: respiratory signs, coagulation disorders, cardiac disease, fever and weakness, but were negative by Baermann analysis.
d) Fifty-eight additional samples were randomly selected from Swiss dogs presented to a veterinary clinic or practice for different reasons.

Sera treatment methods and assays

All 205 sera were tested untreated with the ELISA for detection of circulating *A. vasorum* antigen according to Schnyder et al. [2] and with the sandwich-ELISA for detection of specific antibodies using somatic *A. vasorum* antigen purified with mAb 5/5 [3].

Two different heat treatment methods were initially evaluated. First, samples were tested with a modified heat treatment method described by Little et al. [7]; briefly, samples were heat treated in a dry heat block for 5 minutes at 100 °C and afterward centrifuged for 5 minutes at 13’000 g (heated). Due to almost full coagulation of sera occurring during heat treatment, this method was dismissed due to impracticality. Nevertheless, some samples that contained enough liquid supernatant after heat treatment only were tested and compared with the second method, and comparable results were obtained. This second method, based on heat treatment after addition of EDTA, was slightly modified from Weil et al. [25]: briefly, 0.5M EDTA (pH 8.0) in a dilution of 1:5 was added to samples before they were heated in a dry heat block for 5 minutes at 100 °C and then centrifuged for 5 minutes at 13’000 g. Supernatants were tested with the antigen and antibody ELISAs mentioned above. Samples were also tested with addition of EDTA only, prior to heat treatment, in order to exclude changes in ELISAs due to addition of EDTA.

Antigen cut-off levels for untreated and treated sera were separately defined as mean plus three times standard deviation of optical density (OD) values of the 58 random dog samples.
Seventy-four samples, consisting of 59 samples from experimentally infected dogs, eleven naturally infected dogs and two random samples, were tested with a rapid in-clinic assay (Angio Detect™ Test, IDEXX Laboratories, Westbrook, Maine, USA) according to manufacturer's instructions, both as untreated sera and after EDTA/heat treatment. From experimentally infected dogs samples collected between five and eleven wpi were selected, in order to evaluate the possibility of antigen detection in an earlier phase of infection than actually described [21]. All samples were kept frozen and stored at -20 °C before and after use and treatments.

Statistical analysis

Statistical analysis was undertaken with Microsoft Windows Excel 2007 and IBM SPSS Statistics 22. A one way repeated measure analysis of variance (ANOVA) was conducted to determine differences after treatment applied on sera of experimentally infected dogs. P values of $p<0.05$ were considered statistically significant.

Results

Antigen detection by ELISA in sera from experimentally infected dogs

Sera of dogs collected before inoculation (n=8) and untreated sera of experimentally infected dogs collected prior to three weeks post inoculation (wpi) (n=9) were all antigen negative (Table 1). After three to four wpi single dogs (3/13 samples, 23.1%) started to be positive. Between five and eleven wpi the percentage of positive samples increased over time from 33.3% to 90%. Single dogs were still negative between 12 and 15 wpi. Overall, between five and fifteen wpi, 50.6% (45/89) of the available samples were seropositive. Addition of EDTA only did not alter the outcome of these results.

Table 1: Detection of *Angiostrongylus vasorum* circulating antigen by ELISA in untreated and EDTA/heat treated sera of dogs experimentally infected with *A. vasorum*.

Weeks post inoculation	tested samples n	Untreated, positive sera n (%)	EDTA/heated, positive sera n (%)	Seroconverted negative (-) or positive (+) samples n
-1	8	0 (0)	0 (0)	
1	6	0 (0)	0 (0)	
2	3	0 (0)	0 (0)	
3	8	2 (25)	0 (0)	-2
4	5	1 (20)	0 (0)	-1
5	12	4 (33.3)	3 (25)	-2/+1
6	9	2 (22.2)	2 (22.2)	-1/+1
7	15	4 (26.6)	10 (66.6)	-1/+7
8	14	5 (35.7)	11 (78.6)	+6
9	13	8 (61.5)	12 (92.3)	+4
10	10	8 (80)	9 (90)	+1
11	10	9 (90)	9 (90)	
12 - 15	6	5 (83.3)	5 (83.3)	
Total	119	48 (40.3)	61 (51.3)	-7/+20
Total (5 -15 weeks)	89	45 (50.6)	61 (68.5)	-4/+20

After EDTA/heat treatment, of the 22 samples collected before five wpi none were positive. First positive samples were obtained starting from five wpi, also with increasing percentages over time. However, seven previously positive samples collected between three and seven wpi became negative; instead, additional 20 samples obtained between five and ten wpi became positive following EDTA/heat treatment. Overall, of the 89 samples collected after five wpi, 61 (68.5%) were antigen positive. Particularly between seven and ten wpi additional 34.6% (18/52) became positive.

ANOVA showed that results obtained with EDTA/heat treated samples were significantly different from untreated samples ($p=0.012$).

Antigen detection in sera from naturally infected dogs, from clinically suspect cases and random sera

Of the 18 naturally infected positive dogs, 13 were antigen positive prior to EDTA/heat treatment. After EDTA/heat treatment 15 of 18 sera were positive, three samples remained negative (Fig. 1).

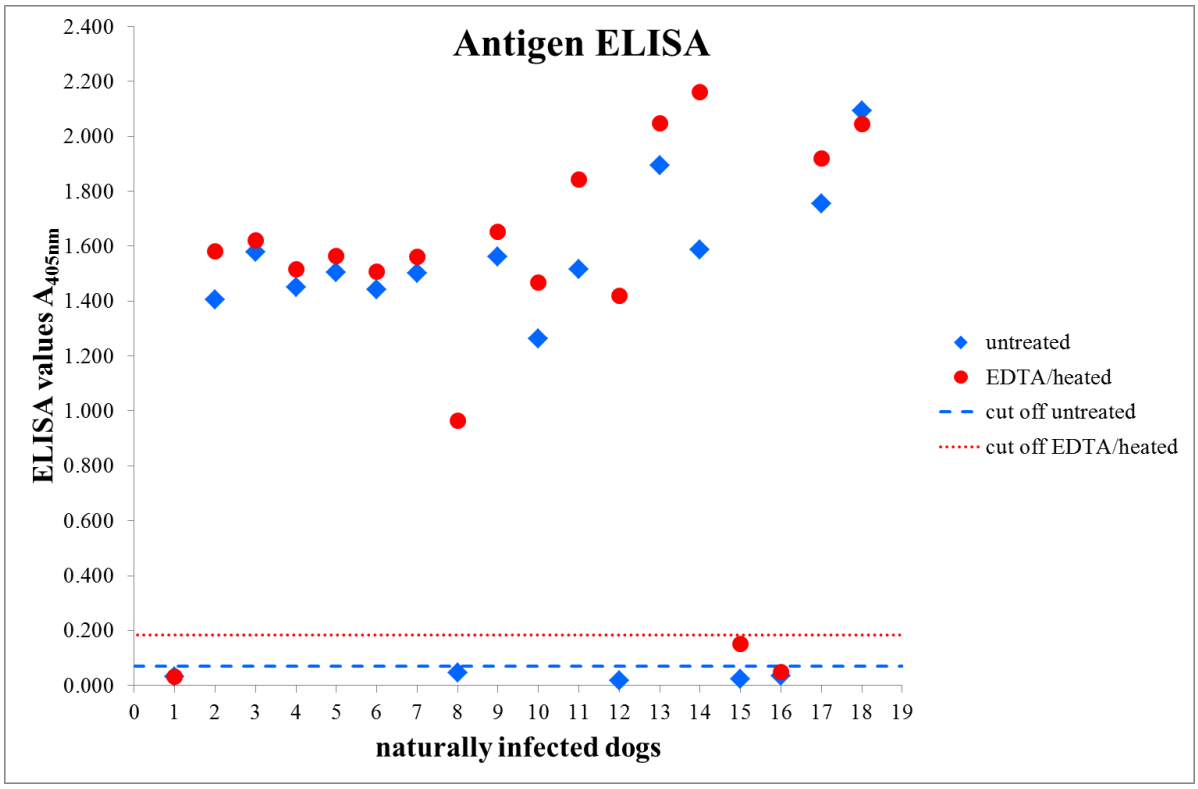

Figure 1: *Angiostrongylus vasorum* antigen optical density values of untreated and EDTA/heat treated sera samples from 18 naturally infected dogs.

Three samples of dogs which were suspect for angiostrongylosis but Baermann negative were initially antigen positive, two of these samples were only marginally above the cut-off. After EDTA/heat treatment only the one sample which was not marginally above cut-off remained positive, with an increase of the OD value (from 0.887 to 1.252).

One of 58 random sera samples was antigen positive prior to treatment (slightly above the corresponding cut-off). After EDTA/heat treatment this single positive sample became clearly negative, however another sample became antigen positive (Fig. 2).

Addition of EDTA only did not alter these results.

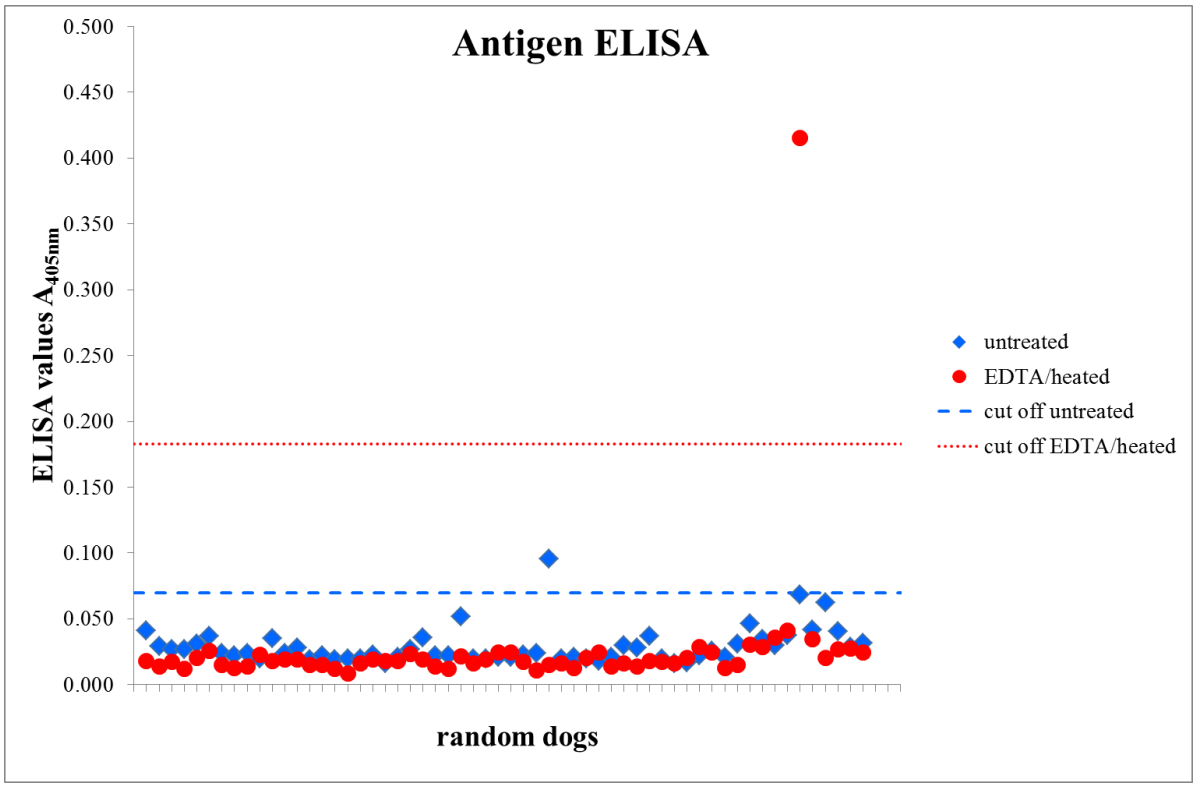

Figure 2: Optical density values of untreated and EDTA/heat treated sera samples from 58 randomly selected dogs tested for circulating *Angiostrongylus vasorum* antigen.

Antibody detection by ELISA

All samples of experimentally infected dogs collected before six wpi (n=42) were antibody negative, all samples collected after six wpi (n=77) were antibody positive. Of these, after EDTA/heat treatment only 27.3% (21 out of 77) remained positive.

In clinic antigen detection assay (Angio Detect™ Test)

Four samples collected 9 (n=2), 11 (n=1) and 13 (n=1) wpi out of the 59 tested sera from experimentally infected dogs were positive for *A. vasorum* antigen in the rapid in-clinic test before and after sera treatments. One additional sample from an experimentally infected dog collected eight wpi became positive after EDTA/heat treatment. The remaining samples were all negative before and after EDTA/heat treatment (Table 2). The same sera tested by ELISA were positive in earlier stages of infection, and by EDTA/heat treatment additional sera were positive: from nine wpi onwards, all were seropositive.

Six sera from eleven naturally infected dogs were antigen positive before and after EDTA/heat treatment. The other five remained negative. The same five Angio Detect™ negative samples were also negative in the ELISA as untreated sera, after EDTA/treatment two of these five negative samples became positive in the ELISA.

The two random sera were both negative before and after EDTA/heat treatment.

Table 2:

Circulating antigen detection in selected serum samples from dogs (n=21) experimentally and naturally (n=11) infected with *Angiostrongylus vasorum* tested by Angio Detect™ in-clinic assay before and after sera EDTA/heat treatment, and corresponding results of untreated and EDTA/heat treated sera tested with the antigen ELISA [2]. Sera between five and eleven weeks were selected to evaluate a possible earlier antigen detection with Angio Detect™ and do not represent all sera tested with the antigen ELISA.

		Experimentally infected dogs			
		In-clinic assay Angio Detect™		Antigen ELISA	
Weeks post inoculation	tested samples n	untreated positive sera n (%)	EDTA/heated positive sera n (%)	untreated positive sera n (%)	EDTA/heated positive sera n (%)
5	4	0 (0)	0 (0)	1 (25)	0 (0)
6	6	0 (0)	0 (0)	2 (33.3)	2 (33.3)
7	11	0 (0)	0 (0)	3 (27.2)	8 (72.7)
8	12	0 (0)	1 (+) (8.3)	5 (41.7)	11 (91.7)
9	10	2 (+/+) (20)	2 (+/+) (20)	6 (60)	10 (100)
10	9	0 (0)	0 (0)	8 (88.9)	9 (100)
11	7	1 (+) (14.3)	1 (+) (14.3)	7 (100)	7 (100)
13	1	1 (++) (100)	1 (++) (100)	1 (100)	1 (100)
15	1[1]	0 (0)	0 (0)	1 (100)	1 (100)
Total	61	4 (6.6)	5 (8.2)	27 (44.3)	49 (80.3)
		Naturally infected dogs			
n.a.	11	6 (54.5)	6 (54.5)	6 (54.5)	8 (72.7)

+ = slight, visible coloration; ++ = good visible coloration [21]

[1]: dog with worm burden = 1 at necropsy

Discussion

Previous evaluations showed that circulating *A. vasorum* antigen detection in untreated sera is possible starting from five wpi, increasing with duration of the infection, and can reach high sensitivity (95.7%) and specificity (94%) [2]. The here presented data with samples obtained under experimental settings confirm these results and show that with heat treatment of sera antigen detection sensitivity by ELISA can be improved between seven and ten wpi: an additional 34.6% of sera became positive. On the other hand, five out of seven previously positive samples collected between three and five wpi turned negative with EDTA/heat treatment, therefore decreasing the sensitivity in this earlier phase of infection, in which also clinical signs pass unnoticed.

An experimentally infected dog harboring a single adult *A. vasorum* specimen became antigen positive only fifteen wpi (on the day of necropsy) in both, the untreated and heat treated sample. This serum was negative with Angio Detect™ indicating that dogs harboring a small number of adult parasites might have antigen values below the threshold, and that antigen detection may not be improved by heat treatment in such cases.

More interestingly, single dogs harboring high numbers of parasites were negative in the antigen detection ELISA: three out of four untreated samples (collected between three and eight wpi) of one dog harboring 165 adult *A. vasorum* specimens [2] were negative, while all samples of this dog collected after five wpi became positive after heat treatment. In combination with a high worm burden, this could be an indication for hyperimmune sera with blocking antibodies preventing antigen to be detected. Immune complex formation was previously assumed to occur in animals infected with *A. vasorum* [26] and has generally been discussed as the reason for low or absent antigen detection, especially in animals infected with *D. immitis* [8, 27]. Like *A. vasorum*, this filarial nematode has dogs as definitive hosts, also resides in their pulmonary arteries and the heart, and is responsible for potential fatal infections. Animals infected with *D. immitis* or *A. vasorum* often suffer from persisting chronic infections that can result in hyperglobulinemia, and therefore may induce antigen-antibody complexes formation [7, 25, 27-29].

Also in sera of proven naturally infected dogs antigen detection sensitivity improved, with two sera becoming additionally positive with EDTA/heat treatment. Results obtained with samples of animals with suspect clinical signs for angiostrongylosis (but negative for faecal L1 detection) furthermore suggest that heat treatment may support the differentiation between positive and negative samples: two samples that were marginally above the cut-off as untreated samples became negative after EDTA/heat treatment. In contrast, the OD value of a third dog with suspect clinical signs increased with heat treatment, possibly confirming a true infection. Similarly, the single positive sample among the random sera being slightly above the corresponding cut-off became clearly negative, while another previously negative became positive with treatment.

On the other side, EDTA/heat treatment of sera may induce loss of specificity of the ELISA. It is likely that heat treatment causes background or interference, and this could therefore decrease specificity, although we adopted a method which is believed to not increase background values [25], by adapting the cut-offs correspondingly.

Other methods than heating, such as acidification of sera, are described. However, in the case of HIV antigen detection, heat treatment was more effective than acidification [30, 31]. We

evaluated heat treatment only and heat treatment with addition of EDTA. Heat treatment only had limitations because sera tended to almost fully coagulate and therefore there was not sufficient liquid supernatant left for testing for most of the sera. If instead samples additionally contained EDTA, coagulation did not occur to such extent and more supernatant could be obtained. Thus, this latter method was used and is consequently recommended.

A very similar heating technique to the here performed methods was recently used by Ciucă et al. [32] to detect *D. immitis* antigen in Romanian stray dogs: detection increased by 18.6% after heat treatment. However, the authors also suggested that heat treatment might have triggered cross reactions with *D. repens* [32]. Similarly, we cannot exclude that heat treatment may have created interference, background or even cross reactions with other parasites in our tests.

The ELISA for detection of specific antibodies can detect *A. vasorum* antibodies as early as 5 wpi. The test can be performed simultaneously with antigen detection: together, antigen and antibody testing have the highest positive predictive value and provide indications on ongoing and previous infections for individual dogs and for population studies as well [5, 33]. In the here presented study, all untreated sera of experimentally infected dogs collected after six wpi were antibody positive. In contrast, after EDTA/heat treatment only 27.3% (n=21) remained seropositive. This is indicating that heating antibodies to 75 °C or higher will induce aggregate formation, as previously described [34]. For instance, heating IgGs to 71 °C will lead to denaturation of both F domains [35]. Since our samples were heated to 100 °C it is very likely that detectable antibodies were destroyed through aggregate formation or denaturation.

The here performed EDTA/heat treatment method did not really improve detection sensitivity of the in clinic assay Angio Detect^{TM}: a single additional sample from an experimentally infected dog collected eight wpi became positive. In a previous study this test was shown to detect *A. vasorum* antigen earliest at nine wpi in dog samples, and after 14 wpi all tested dogs were positive. The evaluations demonstrated that the rapid assay was detecting *A. vasorum* infected dogs with a delay of 3-4 weeks, compared with the ELISA. On the other side, the assay was highly specific (100% specificity) [21]. In that work, single sera of naturally and experimentally infected dogs were already retested after heat treatment: Nine out of twelve sera, which were previously negative in the Angio Detect^{TM}, became positive after heat treatment, improving test sensitivity [21]. Based on these results, we used a slightly different sera EDTA/heat treatment method and particularly focused on experimental sera collected in this window between five and eleven wpi, when more experimental samples were

seropositive by ELISA than with Angio Detect ™. This, together with potential individual differences, may have somewhat influenced the outcome of the results.

Heat treatment methods are easily performed. However, veterinary clinics and practices will most likely not possess the required laboratory equipment, such as heat blocks and high performance centrifuges, and time to invest in such treatments. Therefore, although EDTA/heat treatment may improve detection sensitivity for single samples tested with the in clinic assay, they most likely cannot be performed on site. In addition, due to controversial results observed with randomly selected sera in the antigen detection ELISA, we cannot recommend to pretreat all sera prior to testing. For the ELISAs, considering that heat treatment mostly inhibits antibody detection, the combined antigen and antibody detection in untreated sera still proved to be more reliable and consistent. For questionable antigen results, which occur especially between seven and ten wpi, and, generally, in animals with typical clinical signs of an *A. vasorum* infection and negative antigen and antibody results, it may be an option to pretreat sera. As early diagnosis is essential for appropriate treatment and prevention of complications, repeating the initial testing may be recommended, and will rule out immune complex formation in previously missed *A. vasorum* infections.

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